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Effects of Dietary Addition of Non-Ionic Surfactants on Ruminal Metabolism and Nutrient Digestion of Chinese Merino Sheep

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ABSTRACT

This study was supposed to evaluate the effects of three non-ionic surfactants (NIS) (Tween 40, 60 and 80) on nutrient digestibility and rumen fermentation. Four Chinese Merino rams, fitted with ruminal and duodenal cannulas, were used in a 4×4 Latin square designed experiment. The four treatments were the basal diet (the control) and the basal diet supplemented with 10 g day^{-1} Tween 40, 60 or 80, respectively. Results showed that there were no differences among the four treatments in nutrient intakes. When compared to the control, feeding NIS had no effect on ruminal or total tract digestibility of Organic Matter (OM), Crude Protein (CP), cellulose and hemicellulose. Sheep consuming Tween 60 or 80 tended to have higher apparent ruminal digestion of Dry Matter (DM) ($p = 0.074$). On rumen fermentation characteristics, ruminal pH and protozoa counts were not affected by NIS supplementation. However, carboxymethylcellulase activity was improved by Tween 60 and 80 ($p<0.01$). Sheep feed Tween 40, 60 or 80 resulted in lower ($p<0.05$) ruminal ammonia nitrogen (NH_3-N) and higher ($p<0.01$) total Volatile Fatty Acid (VFA) concentrations. In terms of individual VFA, including Tween 60 or 80 in the diet significantly improved concentrations of acetate ($p<0.01$), propionate ($p<0.01$) and butyrate ($p<0.05$) in ruminal fluid but did not alter the ratio of acetate to propionate. It was concluded that Tween 60 and 80 reduced rumen NH_3-N concentration and increased carboxymethylcellulase activity and total VFA, as well as acetate, propionate and butyrate concentrations but had no effect on nutrient intakes and digestion. It is inferred that potential uses of the Tween 60 and 80 as feed additives for sheep to optimize ruminal fermentation.

Key words: Nonionic surfactants, aliquot, basal diet, rumen metabolism, nutrient digestibility

INTRODUCTION

Crop residues and agro-industrial by-products such as straw of cereals and stover from maize and sorghum usually contain a high proportion of cellulose (Parthasarathy Rao and Hall, 2003). When these fibrous by-products were used as ruminants feed, it is difficult to break down in the rumen (Kamande *et al*., 2000). Considerable efforts have been devoted to optimizing the ruminal environment with the aim of improving roughage utilization. As a result, some feed additives that are capable of influencing fiber fermentation and digestion in ruminants were developed (Lee and Ha, 2003; Patra, 2011).

Over the past several decades, surfactants have been used successfully to enhance enzymatic hydrolysis of fibrous materials in bioethanol production (Zheng *et al*., 2008; Helle *et al*., 1993).

Tween 80, one of non-ionic surfactants (NIS), was found to increase cellulose hydrolysis by increasing cellulase stabilization and preventing inactivation of adsorbed enzymes on substrates (Eriksson *et al*., 2002; Helle *et al*., 1993). In addition, Tween 80 is well known as an effective surfactant that stimulates the release of enzymes from a range of aerobic fungi (Maheshwari *et al*., 2000) and ruminal bacteria (Lee *et al*., 2003; Lee and Ha, 2003). Furthermore, including Tween 80 in mixed cultures of ruminal bacteria increased degradation of cellulose (Hwang *et al*., 2008). Lee *et al*. (2003) reported that Tween 80 might greatly stimulate the growth rate of anaerobic ruminal microorganisms such as noncellulolytic bacteria and fungi. Dietary addition of Tween 80 for dairy cows increased milk production by improving feed efficiency (Shelford and Kamande, 2001; Lee *et al*., 2003). Ahna *et al*. (2009) demonstrated that Tween 80 improved ruminal fermentation of fibrous parts of feeds and consequently enhanced performance of beef steers fed a high-roughage diet. Although Tween 80 has been shown to alter the cattle ruminal fermentation favorably, few researchers have attempted to determine if Tween 40 or 60 would elicit a similar response in the sheep rumen, with consequent improvements to its production.

The present study was conducted to assess the effects of dietary addition of Tween 40, 60 and 80 on the metabolism in rumen and nutrient digestibility of Chinese Merino sheep.

MATERIALS AND METHODS

Sheep and diets: Four Chinese Merino rams (2-year-old, initial body weight of 43±6 kg) fitted with ruminal and duodenal cannulas were assigned randomly to a 4×4 Latin square design. The four treatments were the basal diet (control) and the basal diet supplemented with 10 g day^{-1} Tween 40, 60 or. All animals were maintained in individual pens (1×1.2 m, 0.8 m high) at experimental sheep farm of Xinjiang Agricultural University (Urumqi, China) with free access to water. The surfactants were dosed daily directly mixed into the concentrate. The concentrates (Table 1) were fed at 436.5 g per day in 2 equal allotments at 09:00 and 21:00. Corn stover was fed *ad libitum* and water was available at all times. Each treatment period was conducted for 26 days, comprising 14 days of adaptation to the diet, followed by 3 days for rumen sample collection, 3 days for duodenal digesta collection and 6 days for feces collection. During the last 6 days, corn stover intake and feed residues were recorded daily. This experiment was conducted from May 18, 2009 to October 28, 2010.

Sampling and chemical analyses: On day 15 to 17 of the treatment period, 100 mL of ruminal contents was obtained via the ruminal cannula from each sheep before morning feeding and at 1.5, 4, 8 and 12 h after feeding. The pH of ruminal content was determined immediately with a pH-meter (model FE20; METTLER TOLEDO, China). Samples were strained through four layers of cheesecloth and the filtered liquid were stored at -20°C for Volatile Fatty Acids (VFA) and carboxymethylcellulase (EC 3.2.1.4) determination. A 10 mL aliquot of the filtered liquid was added with 0.2 mL saturated mercuric chloride and stored at -20°C for protozoa counting. A portion of 2 mL ruminal fluid was acidified with 8 mL of 0.2 M hydrochloric acid and stored at -20°C for ammonia nitrogen (NH_3-N) analysis.

Carboxymethylcellulase activity in ruminal fluid was determined as described by Lee and Ha (2003) with modifications. In brief, 10 mL ruminal fluid was sonicated for 80 repetitions (ultrasonication power 400 W) with 3 sec of ultrasonication and 4 sec of interval on ice using a sonicator (model JY92-II; Ningbo Scientz Biotechnology Co., China) to disrupt microbial cells. The sample was then centrifuged at 20 000×g (4°C) for 15 min and the supernatant was used as the

Table 1: Ingredient and chemical composition of the concentrate supplement and corn stover (DM basis)

Ingredient	Concentrate supplement (%, DM)	Chemical composition	Concentrate supplement (g kg^{-1}, DM)	Corn stover (g kg^{-1}, DM)
Yellow corn, ground	68.45	DM (g kg^{-1})	910.0	925.6
Cotton seed meal	28.00	CP	212.7	66.0
Salt	2.30	Crnde ash	59.0	105.8
Urea	0.80	Cellulose	38.4	356.4
Calcium phosphate	0.40	Hemicellulose	74.0	257.7
Premix*	0.05	Calcium	4.8	5.3
		Phosphorus	4.6	0.8

*Contained; I: 0.75 g, Se: 0.45 g, Co: 0.3 g, Cu: 1.27 g, S: 23 g, Vitamin A: 4000 KIU kg^{-1}, Vitamin D: 800 KIU kg^{-1}

carboxymethylcellulase source. The enzyme activity against sodium carboxymethyl cellulose (CMC-Na) was determined by incubating 0.5 mL of supernatant with 1 mL 1% (w/v) CMC-Na in 0.2 M disodium hydrogen phosphate-citric acid buffer (pH 5.5). After 10 min incubation at 39°C, the reaction was stopped by adding 1.5 mL dinitrosalicylic acid. Aliquot was mixed and bathed in boiling water for 5 min and then centrifuged at 570×g for 10 min and the total amount of reducing sugars in the supernatant was determined colorimetrically by Dinitrosahcylic acid method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μg of glucose equivalent per minute under the conditions described above.

Samples for VFA analysis were centrifuged at 20 000×g for 15 min at 4°C. Subsamples of supernatant after centrifugation were processed as described by Samuel *et al.* (1997) for analysis of VFA by HPLC (model LC-6A; SHIMADZA Corporation, Kyoto, Japan). NH_3-N was measured by the indophenol method (Weatherburn, 1967). The ruminal protozoa numbers were counted by an improved counting cell (0.5 mm depth) with Methylgreen-Formalin-Saline (MFS) solution (Boyne *et al.*, 1957).

For the daily duodenal digesta flow calculation, on day 18 to 20 of the treatment period, approximately 80 g of duodenal digesta was collected at 6-h intervals starting at 09:00. Collection times were adjusted ahead 2 h daily so that by the end of the each sampling period, 12 samples were collected at every even hour of the 24-h day. Duodenal samples were composited by animal for each collection period. All the composited samples were dried in oven at 65°C to constant weight for chemical analysis.

On day 21 to 26 of the treatment period, total fecal output for each sheep was collected using fecal collection bag and an aliquot (10%) of total fecal output was subsampled each day for digestibility determination. Daily subsamples of feces within animal were pooled to form composite samples and dried in oven at 65°C to constant weight before chemical analysis.

All the samples, including dried duodenal digesta, feces and weekly collected samples of offered concentrate and corn stover, were ground to pass through a 1mm screen and used to measure nutrient contents. Dry Matter (DM), ash, Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), Acid Detergent Lignin (ADL) and Crude Protein (CP) were measured as AOAC (1999) described. Hemicellulose percentage was calculated as NDF (%) -ADF (%) and cellulose percentage was calculated as ADF (%) -ADL (%).

Calculations and statistical analysis: To determine the duodenal digesta flow, ADL was used as an indigestible marker of digesta. Nutrients flow at duodenum and apparent ruminal digestion of nutrients were calculated as Lu and Xie (1991) described.

The data were subjected to the analysis of variance for a 4×4 Latin square design using the General Linear Models (GLM) of PASW Statistics 18.0 (Norušis, 2009) as:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

where, y_{ijk} is an observation; μ is the overall mean; α is the fixed effect of NIS supplementation ($j = 1, 4$); β is the fixed effect of animal ($i = 1, 4$); γ is the fixed effect of treatment periods ($k = 1, 4$); ε_{ijk} is the residual error. Significance of differences between treatments was declared significant at $p \leq 0.05$ and multiple comparisons among treatment means were performed by Duncan's New Multiple Range Test.

RESULTS

Effects of NIS on the voluntary intake and ruminal digestion of nutrients: Effects of dietary addition of Tween 40, 60 and 80 on voluntary corn stover, nutrient intakes are summarized in Table 2. There were no differences in corn stover intake among groups. As planned, concentrate intakes were the same among treatments because sheep were fed restricted amounts. Compared to the control, the Tween 80 group had a tendency for an increase consumption of the corn stover and nutrients.

DM flow at duodenum of Tween 60 group was lower than that of control. The OM, CP, cellulose and hemicellulose flow at duodenum were not affected by NIS supplementation.

Sheep consuming Tween 60 or 80 tended to have higher apparent ruminal digestion of DM ($p<0.1$), but the digestibility on of OM, CP and cellulose, as well as hemicellulose were almost similar among treatments (Table 2).

Table 2: Effects of NIS on voluntary intake and ruminal digestion of nutrients of sheep

Item	Control	Tween 40	Tween 60	Tween 80	SEM	p-value
Intake (g day^{-1})						
Concentrate	436.5	436.5	436.5	436.5	-	-
Corn stover	1026.5	1027.4	1008.7	1072.1	34.59	0.213
DM	1313.2	1313.0	1296.0	1354.9	32.06	0.209
OM	1293.9	1294.4	1278.8	1334.8	30.90	0.228
CP	144.9^{ab}	145.0^{ab}	143.1^{b}	149.0^{a}	2.31	0.069
Cellulose	382.4	381.9	377.6	400.0	12.26	0.181
Hemicellulose	294.2	293.4	290.0	306.0	8.93	0.250
Flow at dnodenum (g day^{-1})						
DM	837.9^{a}	819.0^{ab}	777.5^{b}	805.4^{ab}	22.48	0.156
OM	716.9	726.0	680.3	701.0	17.15	0.322
CP	116.9	117.0	111.4	115.5	4.68	0.226
Cellulose	176.4	168.3	173.8	166.9	7.14	0.844
Hemicellulose	144.5	114.5	131.4	140.1	6.05	0.580
Apparent ruminal digestion (% of intake)						
DM	36.4^{b}	37.6^{ab}	40.0^{a}	40.3^{a}	0.94	0.074
OM	44.6	43.9	46.7	47.2	0.98	0.193
CP	19.9	19.6	22.3	22.8	2.41	0.270
Cellulose	54.1	55.7	53.8	57.7	1.72	0.655
Hemicellulose	51.1	51.1	54.2	54.7	1.15	0.501

[b]Values in the same row with different superscripts differ ($p<0.05$), DM: Dry matter, CP: Crude protein, OM: Organic matter

Table 3: Effects of NIS on average daily ruminal pH, fermentation end products, carboxymethylcellulase activity and protozoal counts

Item	Control	Tween 40	Tween 60	Tween 80	SEM	p-value
Ruminal pH	6.24	6.19	6.16	6.10	0.03	0.181
Carboxymethylcellulase activity (U mL^{-1})	96.88^{b}	100.60^{b}	108.54^{a}	113.65^{a}	2.06	<0.01
Protozoal counts ($\times 10^{4}$ mL^{-1})	33.78	34.25	36.16	33.38	1.82	0.517
NH_3-N (mmol L^{-1})	11.90^{a}	11.10^{b}	11.18^{b}	10.98^{b}	0.18	0.013
Total VFA (mmol L^{-1})	94.49^{c}	99.51^{b}	102.29^{b}	106.78^{a}	1.37	<0.01
Acetate (mmol L^{-1})	63.35^{c}	66.65b^{c}	68.38^{ab}	71.07^{a}	0.92	<0.01
Propionate (mmol L^{-1})	20.03^{c}	21.15b^{c}	21.86^{ab}	22.88^{a}	0.35	0.012
Butyrate (mmol L^{-1})	11.10^{c}	11.70b^{c}	12.06^{b}	12.83^{a}	0.23	<0.01
Acetate: Propionate	3.18	3.15	3.13	3.11	0.03	0.947

^{c}Values in the same row with different superscripts differ ($p<0.05$)

Table 4: Apparent nutrient digestibilities in total digestive tract of sheep fed with different NIS

Item (%)	Control	Tween 40	Tween 60	Tween 80	SEM	p-value
DM	61.47	63.35	63.67	63.92	0.76	0.325
OM	61.61	63.69	63.99	64.36	0.79	0.246
CP	63.84	66.42	66.32	65.79	1.25	0.389
Cellulose	55.17	57.75	57.47	59.63	0.77	0.264
Hemicellulose	54.03^{b}	58.31^{ab}	56.35^{ab}	58.74^{a}	0.96	0.108

^{b}Values in the same row with different superscripts differ ($p<0.05$), DM: Dry matter, CP: Crude protein, OM: Organic matter

The effects of NIS on ruminal fermentation characteristics: The effects of NIS on ruminal fermentation characteristics are shown in Table 3. As compared to the control, average daily carboxymethylcellulase activities were increased by Tween 60 and 80 ($p<0.05$). Protozoal counts were not affected by NIS Supplementation. Tween 40, 60 or 80 lowered ruminal NH_3-N ($p<0.05$). The concentrations of total VFA were increased by NIS addition ($p<0.01$). The highest one was Tween 80, averaged 106.78 mmol L^{-1} and the lowest one was the control, averaged 94.49 mmol/l. In terms of individual VFA, including Tween 60 and 80 in the diet resulted in higher concentrations of acetate ($p<0.01$), propionate ($p<0.01$) and butyrate ($p<0.05$) in ruminal fluid, while those of Tween 40 group were intermediate between treatments of control and Tween 60. The ratio of acetate to propionate was not affected by these NIS.

Effects of NIS on apparent nutrient digestibility: Effects of different NIS on the apparent nutrient digestibilities in total digestive tract of sheep are shown in Table 4. Apparent digestibilities of DM, OM, CP and cellulose were not different among treatments. As compared with the control, the apparent digestibility of hemicellulose tended to be increased by Tween 80 treated ($p = 0.108$).

DISCUSSION

Few attempts were conducted to evaluate the effects of Tween 40, 60 and 80 on the voluntary intake of sheep. According to present results, the voluntary intakes of concentrate, corn stover, as well as nutrients were unaffected by Tween 40, 60 or 80. McAllister *et al.* (2000) and Hristov *et al.* (2000) reported that intakes of DM, NDF, ADF and CP of lambs or steers were unaffected by Tween 80 addition. Present results are in agreement with their findings. Ahna *et al.* (2009) found that low dosage of Tween 80 (15 g day^{-1}) did not effect in the roughages intake of steers. However, when Tween 80 addition increased to 30 g day^{-1}, intake of corn silage and rice straw was higher ($p<0.05\%$) than the control. These results implied that the effect of Tween 80 on the intake may dosage dependent.

In the present research, apparent ruminal DM digestion tended to be improved by Tween 80 and this agree with previously reported studies (Lee and Ha, 2003; Lee *et al.*, 2003, 2007). This may due to an increasing of carboxymethylcellulase activity after Tween 80 addition.

Present results confirm those of Baah *et al.* (2005), who found that total tract digestibility coefficients of DM, nitrogen, NDF and ADF were not affected ($p>0.05$) by Tween 80 treatment. Kim *et al.* (2004) found that although digestibility of crude fiber increased in Hanwoo steers fed a diet supplemented with 10 g day^{-1} Tween 80, digestibilities of DM, CP, NDF and ADF were unaffected when compared to the control. Furthermore, those authors observed that increasing concentration of Tween 80 tended to increase the digestibility of the above nutrients (Ahna *et al.*, 2009).

Little attention has been paid to the effect of Tween 40 and 60 on the nutrients intake and digestion of sheep which was found to be inefficiency throughout the trial. This may due to the dosage was unsuitability for Tween 40 and 60 or the difference of molecular structure and physico-chemical properties. To our knowledge, this is the first time the effect of Tween 40 and 60 on the nutrients intake and digestion of sheep was investigated. In order to better understand their roles in rumen, therefore, further more studies are needed.

A number of *in vitro* studies have shown that the major effect of Tween 80 on ruminal fermentation was to increase fibrous degradation enzyme activity (Lee *et al.*, 2003; Lee and Ha, 2003; Wang *et al.*, 2004). In the present study, carboxymethylcellulase activities were also improved by Tween 60 and 80 addition but not by Tween 40. Kamande *et al.* (2000) also found that Tween 60 was effective for enhancing rumen microbial protease and cellulase activities. One of the mechanisms of carboxymethylcellulase activity being improved by Tween 80 is to prevent inactivation of enzymes in rumen. Surfactants have been shown to prevent deactivation of cellulase in culture filtrates from *Trichoderma reesei* (Kim *et al.*, 1982). Stabilization of the cellulase activity was attributed to reduced denaturation of the enzyme at the air-liquid interface, due to the surfactant's effectiveness at excluding the enzyme from the interface (McAllister *et al.*, 2000). Another mechanism is to promote ruminal microorganisms to release more enzymes. Yazdi *et al.* (1990) demonstrated that the secretion of several fibrolytic enzymes from *Neurospora crassa* was intimately linked to anaerobic microbial cell membrane lipid composition. An increased fibrolytic enzyme activity from Tween 80 supplementation could be explained by altering membrane fluidity and permeability, thus permitting more enzymes to be released (Yazdi *et al.*, 1990; Lee and Ha, 2003).

Rumen microorganisms produce VFA and gas as a result of their metabolic processes. In the present study, surfactant addition did change VFA concentrations and this agrees with other *in vitro* (Wang *et al.*, 2003; Ahna *et al.*, 2009; Cong *et al.*, 2009) as well with some *in vivo* studies (Kim *et al.*, 2004; Ahna *et al.*, 2009). However, Hristov *et al.* (2000) reported that total ruminal concentrations of VFA and relative concentrations of individual VFA were not affected by Tween 80 treatment. Kim *et al.* (2004) suggested that the effects of Tween 80 on the ruminal fermentation were diet dependent, expressed either through altering the species composition of the rumen microbial population or through altering the interaction between the enzymes and the target substrates. Thus, the response of ruminal fermentation to NIS may relate to NIS dose and type and to the fermented substrate.

The present study has found that NIS could lower the ruminal NH_3-N. Results from previous reports concerning NIS, however, are variable. Ruminal concentration of NH_3-N was unaffected when Tween 80 added to diets (Hristov *et al.*, 2000; Kim *et al.*, 2004; Baah *et al.*, 2005). Nevertheless, Ahna *et al.* (2009) showed that ruminal concentration of NH_3-N was increased

($p<0.05$) by 2 or 4 g day^{-1} Tween 80 supplementation. In our trial, on the contrary, the concentrations of NH_3-N were lowered by the three kinds of NIS. Bach *et al.* (2005) considered that the NH_3-N levels were lower in acidic conditions because of reduced proteolytic activity of the microbes selected at the low pH which has been demonstrated in ruminal *in situ* and *in vitro* studies. In the present experiment, the ruminal pH ranged from 6.10 to 6.19 which was lower than that of Ahna *et al.* (2009) reported. Therefore, the difference of change pattern in NH_3-N may result from the variance in ruminal pH. In addition, ammonia is the preferred source of nitrogen for most rumen bacteria. Then, lowered NH_3-N suggested that higher ammonia assimilation by ruminal microorganisms when NIS was incorporated in diet.

Including NIS tended to have lower ruminal pH than the control. The results from the present experiment agree with similar studies conducted by McAllister *et al.* (2000). Hristov *et al.* (2000) reported that ruminal pH was not altered by Tween 80 (2 g kg^{-1} feed) in steers fed a diet containing 70% barley. Baah *et al.* (2005) also showed that non-lactating Holstein cows receiving 2 mL kg^{-1} TMR of Tween 80 tended to have lower ruminal pH. Contrary to the above observations, Ahna *et al.* (2009) reported that ruminal pH of steers fed 2 or 4 g day^{-1} Tween 80 was lower ($p<0.05$) than that of steers with no supplementation. It is known that the accumulation of acids (most of which are VFAs) in the rumen contribute to its acidic condition and thus can be considered indicative of a decline in rumen pH of Tween 80 treated animals.

Ruminal pH was identified as critical for maintaining fiber digestion (Hiltner and Dehority, 1983). To achieve optimal cellulolytic digestion, a pH of 6.7 (±0.5 pH units) is required and deviations on either side of this are inhibitory (Van Soest, 1994). The cellulolytic, hemicellulolytic and pectinolytic organisms will be inhibited when ruminal pH below 6.2 (Van Soest, 1994). Accordingly, ruminal fermentation and digestion are restrained (Grant and Mertens, 1992). The ruminal pH observed in this study were relatively low considering the ideal values and approaching the suggested minimal pH of 6.2, below which fibrolytic microorganisms are inhibited. This may one of the reasons why the differences of fibrous material digestibilities were not observed among treatments in the present study.

CONCLUSION

In conclusion, results obtained in the present study showed that the effects of the nonionic surfactants Tween 60 and 80 reduced rumen NH_3-N levels and increased carboxymethylcellulase activities and concentrations of total VFA, as well as acetate, propionate and butyrate, but had no effect on nutrient intakes and digestion. Significant effects of Tween 40 on ruminal fermentation, nutrients intake and digestion were not observed. These observations indicate that potential uses of Tween 60 and 80 as feed additives for sheep to improve ruminal fermentation.

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